IMMUNOGLOBULIN RESPONSES OF NORTHERN ELEPHANT AND PACIFIC HARBOR SEALS NATURALLY INFECTED WITH OTOSTRONGYLUS CIRCUMLITUS

Authors: Elson-Riggins, Jocelyn G., Riggins, Scott A., Gulland, Frances M. D., and Platzer, Edward G.

Source: Journal of Wildlife Diseases, 40(3) : 466-475

Published By: Wildlife Disease Association

URL: https://doi.org/10.7589/0090-3558-40.3.466

Journal of Wildlife Diseases, 40(3), 2004, pp. 466–475

IMMUNOGLOBULIN RESPONSES OF NORTHERN ELEPHANT AND PACIFIC HARBOR SEALS NATURALLY INFECTED WITH *OTOSTRONGYLUS CIRCUMLITUS*

Jocelyn G. Elson-Riggins,[1,2,5] Scott A. Riggins,[3] Frances M. D. Gulland,[4] and Edward G. Platzer[2]

[1] Department of Biology, University of California, Riverside, California 92521, USA
[2] Department of Nematology, University of California, Riverside, California 92521, USA
[3] Department of Neurosciences, Deaconess Billings Clinic, 2825 Eighth Avenue North, Billings, Montana 59107, USA
[4] The Marine Mammal Center, Golden Gate National Recreation Area, Marin Headlands, Sausalito, California 94965, USA
[5] Corresponding author (jelsonriggins@msubillings.edu)

ABSTRACT: Immunoglobulin (Ig) binding patterns of Pacific harbor seals (PHS, *Phoca vitulina richardsi*) and northern elephant seals (NES, *Mirounga angustirostris*) to tissues of adult *Otostrongylus circumlitus* were examined by immunoblotting to investigate the role of age in the unusual response of juvenile NES to infection with *O. circumlitus.* Serum was taken from NES between March 1997 and March 2001 and from PHS between May 1996 and August 1999. The serum of seals infected with *O. circumlitus* contained antibodies that bound to all nematode tissues examined. Intensity of band staining on Western blots suggested that there were higher levels of antibody recognizing the excretory-secretory (ES) glands in the serum of NES that were 1 yr and older and in the majority of PHS compared with that in 2- to 9-mo-old NES. All juvenile NES infected with *O. circumlitus* and a proportion of the PHS and older NES infected with *O. circumlitus* contained Ig specific to a 28 kDa protein band that was dominant in the female reproductive tract of the nematode. The Ig binding patterns of NES and PHS to adult *Parafilaroides* sp., larval *Pseudoterranova* sp., and larval and adult *Anisakis* sp. differed sufficiently from that of *O. circumlitus* that immunoblotting for the 28 kDa protein could be useful for diagnosis of this parasite in juvenile NES. The banding patterns suggest that *O. circumlitus* nematodes die and disintegrate in PHS and NES and that NES of 1 yr and older and most PHS respond differently to the ES glands than 2- to 9-mo-old NES.

Key words: Immunoglobulin, lungworm, nematode, northern elephant seal, *Mirounga angustirostris, Otostrongylus circumlitus,* Pacific harbor seal, *Phoca vitulina richardsi.*

INTRODUCTION

Otostrongylus circumlitus (Nematoda: Metastrongyloidea) is principally a lungworm of seals under 1 yr of age with a circumpolar distribution (Onderka, 1989; Measures, 2001). It has an indirect life cycle, with third-stage larvae developing in American plaice (*Hippoglossoides platessoides*) exposed experimentally to first-stage larvae (Bergeron et al., 1997). This nematode is well recognized as a cause of morbidity and mortality in phocids, and in some species severity of disease is proportional to intensity of infection (Onderka, 1989; Vercruysse et al., 2003). In California (USA), *O. circumlitus* is a significant factor in the mortality of Pacific harbor seals (PHS, *Phoca vitulina richardsi*) and northern elephant seals (NES, *Mirounga angustirostris*) that strand along the central coast (Gulland et al., 1997). Pacific harbor seals form discrete subpopulations with a continuous distribution from California to Alaska (USA) and are resident year round in their range, whereas NES breed in California and migrate north twice per year (Riedman, 1990).

Northern elephant seals appear to be less well adapted to the parasite than PHS, because the severity of disease in this species is not proportional to the intensity of infection, and mortality often occurs during the prepatent period, thus preventing parasite reproduction in the host (Gulland et al., 1997). Mortality of juvenile NES may result from pulmonary arteritis and disseminated intravascular coagulation (DIC) with as few as 20 parasites (Gulland et al., 1996, 1997), yet adult NES may have asymptomatic infections with para-

sites in the airways (Gulland, pers. obs.). Characteristics of infection in PHS are, however, typical of those in other host species and include bronchitis and pulmonary arteritis (Gulland et al., 1997; Measures 2001). Due to mortality during the prepatent period, *O. circumlitus* infection is difficult to diagnose ante mortem in NES. The clinical signs are nonspecific in this host, and the presence of larvae in the feces cannot be used to diagnose infection. An effective diagnostic test for *O. circumlitus* in young NES is required so that appropriate treatment and prophylaxis can be developed and evaluated in captive animals and those being rehabilitated.

To investigate whether age was a factor in the unusual NES response to *O. circumlitus*, currently available techniques were used to detect immunoglobulin (Ig) to different tissues of *O. circumlitus* in serum of two different age classes of NES infected with *O. circumlitus*. The Ig binding patterns of these animals were then compared with the Ig binding pattern of PHS infected with *O. circumlitus*.

MATERIALS AND METHODS

Parasite and serum collection

Adult nematodes were obtained from stranded seals during postmortem examination at The Marine Mammal Center (TMMC), Sausalito, California, between May 1997 and July 2001. Some of these animals had been euthanized because the chances of survival were determined to be minimal. This was done by intravenous injection of pentobarbital (389 mg/ml; Anthony Products, Arcadia, California). The nematodes were then rinsed in 0.15 M saline and stored at −80 C.

Blood samples were taken from the extradural intravertebral sinus of live animals by the veterinary staff at TMMC and Sea World, San Diego, California. All animals at Sea World were alive. If an animal at TMMC had recently died, blood was obtained by cardiac puncture. Blood was collected in Vacutainers containing serum separation gel (Becton-Dickinson and Company, Franklin Lakes, New Jersey, USA) and centrifuged at 2,000 × G for 30 min to obtain serum. This was stored at −80 C until used. Serum was taken from NES between March 1997 and March 2001 and from PHS between May 1996 and August 1999.

Nematode extract preparation

Otostrongylus circumlitus were dissected in 0.15 M saline that had been chilled to 4 C. Intestine, excretory-secretory (ES) glands, male and female reproductive tract, and body wall were excised, rinsed in fresh saline, and stored at −80 C until extracts were prepared. Whole nematodes (*O. circumlitus, Parafilaroides* sp., *Pseudoterranova* sp., and *Anisakis* sp.) and *O. circumlitus* reproductive tracts were homogenized in phosphate buffered saline (PBS; Maizels et al., 1991) containing a general use protease inhibitor cocktail (Sigma-Aldrich Corporation, St. Louis, Missouri, USA). Homogenization was performed on ice for 5 min in Eppendorf tubes using disposable grinders (Bel-art Products, Scienceware, Pequannock, New Jersey, USA). One microliter of each homogenate was placed on microscope slides and checked for full maceration by light microscopy. The homogenate was then centrifuged at 16,000 × G for 30 min at 4 C. The supernatant was removed and further centrifuged at 16,000 × G for 30 min at 4 C. This procedure was repeated until no more pellet was obtained. The supernatant was then stored at −80 C.

Extracts of *O. circumlitus* ES gland and intestine were prepared as above, except that they were homogenized in ripa buffer (150 mM NaCl, 50 mM trizma base, 5 mM ethylenediaminetetraacetic acid [EDTA], 1% nonidet P-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], pH 7.5) containing the protease inhibitor mentioned above. *Otostrongylus circumlitus* body wall extracts were homogenized in PBS as above. However, the homogenate was then sonicated on ice at 50 W in a Virsonic 50 Disrupter (Virtis Company, Inc., Gardiner, New York, USA) for 2 min. This was further homogenized on ice for 5 min before centrifuging at 13,800 × G for 10 min at 4 C. The supernatant was labeled F1 and stored at −80 C. The pellet was boiled in PBS containing 1% SDS for 2 min. This was then centrifuged at 13,800 × G for 10 min at room temperature. This supernatant was labeled F2 and frozen at −80 C. The F2 pellet was then boiled in PBS containing 1% SDS and 5% β-mercaptoethanol for 2 min. This was centrifuged at 13,800 × G for 10 min at room temperature. The supernatant was labeled F3 and stored at −80 C.

The protein content of each extract and serum sample was estimated by either Bio-Rad or Detergent Compatible (DC) protein assays (Bio-Rad, Hercules, California, USA) using a Benchmark microplate reader (Bio-Rad) with bovine serum albumin as a standard.

SDS-PAGE and immunoblotting

Protein extracts were boiled in Laemmli sample buffer (Laemmli, 1970) for 10 min and separated by SDS polyacrylamide gel electrophoresis (PAGE) on either 4% stacking and 12% separating gels (Doucet and Trifaró, 1988) or 4–20% tris-HCl precast gels (Bio-Rad). For immunoblotting, proteins were transferred to Millipore Immobilon-P transfer membranes (Millipore Corporation, Bedford, Massachusetts, USA) using a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad), dried according to the manufacturer's instructions and stored at −20 C until use. The membranes were then wetted with 100% methanol and rinsed first in nanopure water, then in TBT (20 mM trizma base, 500 mM NaCl, pH 7.5, with 0.05% Tween-20). They were then blocked with TBT containing 5% dry skim milk (Becton Dickinson and Company, Sparks, Maryland, USA) for 1 hr. After this treatment and each subsequent treatment the membranes were rinsed in TBT. They were incubated for 1 hr in antibody buffer (TBT containing 1% skim dry milk) containing serum of the appropriate concentration, which was determined in previous experiments. They were then incubated for 1 hr in horseradish peroxidase linked protein A (protein A-HRP; Amersham Pharmacia Biotech, Arlington Heights, Illinois, USA) diluted to 4 ng/ml in antibody buffer, and the proteins were detected by enhanced chemiluminescence (ECL; Amersham) according to the manufacturer's instructions. The membranes were then incubated for 1 hr in 0.015 μg/ml HRP conjugated streptavidin (Pierce, Rockford, Illinois, USA) followed by ECL to detect the standards. Afterward, selected membranes were rinsed and stripped using a Re-Blot Plus Western Blot Recycling Kit (Chemicon International, Temecula, California, USA) according to the manufacturer's instructions. These membranes were then blocked and incubated in serum and protein A-HRP before the detection processes described above.

Four micrograms of protein per well were loaded for immunoblots of extracts of *O. circumlitus* using Bio-Rad Mini-Protean II and Mini-Protean 3 electrophoresis cells (Bio-Rad) and 15 well combs. These blots were probed with serum at a concentration of 0.1 μg protein/μl for NES and 0.06 μg protein/μl for PHS. These sera concentrations were selected to optimize immunoblot conditions. Two micrograms of protein per well were loaded for the immunoblots testing different nematode genera and serum was used at a concentration of 0.2 μg/μl protein for NES and 0.12 μg/μl protein for PHS. Biotinylated SDS-PAGE standards (Bio-Rad; 0.26 μg protein/well) were used to determine molecular weights. Full range rainbow markers (Amersham) were also run on each gel to determine when to terminate electrophoresis. This marker lane and lanes not directly pertaining to the results were digitally removed from the figures.

Study design

The majority of animals were classed as infected with *O. circumlitus* if these nematodes were detected in airways, heart, or pulmonary arteries at necropsy. However, three of the yearling NES were diagnosed as infected with *O. circumlitus* by fecal examination for *O. circumlitus* larvae. Two of these animals were treated to remove nematodes, recovered from clinical signs of infection, and were subsequently returned to the wild. A seal was classed as uninfected if no *O. circumlitus* were detected at necropsy, no *O. circumlitus* larvae were detected in feces, and it had a feeding history consistent with lack of exposure (while in captivity seals were fed frozen fish assumed free of live nematodes). Parasites of species other than *O. circumlitus* were not detected in uninfected animals. However, infected animals had the following histories with respect to other parasites: of the 10 NES infected with *O. circumlitus* that were age class 2–9 mo, *O. circumlitus* alone was detected in five animals, *Parafilaroides* sp. in one, anisakids in four, tapeworms in three, and flukes in one; of the seven NES infected with *O. circumlitus* that were age class 1–2 yr, *O. circumlitus* alone was detected in two animals, *Parafilaroides* sp. in one, anisakids in three, tapeworms in two, flukes in five, and acanthocephalans in one; of the 12 PHS infected with *O. circumlitus* that were examined, *O. circumlitus* alone was detected in five animals, *Parafilaroides* sp. in three, anisakids in four, flukes in one, and acanthocephalans in five. Animals from TMMC were fed thawed previously frozen herring, and animals from Sea World were fed thawed previously frozen herring, sardines, and capelin.

The *O. circumlitus* extract gels were probed with serum from the following classes of NES infected with *O. circumlitus* (all from TMMC): nine animals 2–6 mo of age, one 9-mo-old animal, and seven 1- to 2-yr-old animals (Table 1). The *O. circumlitus* extract gels were also probed with serum from four uninfected NES. Three of these uninfected seals were 2 mo old and had been in captivity prior to weaning. The fourth uninfected NES was taken into captivity at weaning age and died 4 days later. Gels were also probed with serum from 12 PHS infected with *O. circumlitus*, all from TMMC, ranging

TABLE 1. Mean band intensity of immunoblot staining for *Otostrongylus circumlitus* infected and uninfected northern elephant and Pacific harbor seals of different age classes.

Seal species	Infection[b]	Age class	*n*	Mean band intensity[a]	
				28 kDa[c]	ES gland[d]
Northern elephant seal	−	2 mo	4	0	0
	+	2–6 mo	9	2.4	1.0
	+	1–2 yr	7	1.0	3.4
Pacific harbor seal	−	1.5 mo–18 yr	6	0.5	0.5
	+	1 mo–4+ yr	12	0.2	2.2

[a] Mean banding intensity based on a visual scale from 0 (no binding) to 4 (very strong binding).
[b] Negative (−) or positive (+) for *Otostrongylus circumlitus* infection.
[c] 28 kDa band in the female reproductive tract of *Otostrongylus circumlitus*.
[d] Excretory-secretory (ES) gland of *Otostrongylus circumlitus*.

in age from 4 to 6 wk to one adult of over 4 yr of age. Serum from six uninfected PHS was also tested. Four were seals that had been born in captivity and raised at Sea World, and two were animals from TMMC. The animals from Sea World ranged from 6 mo to 18 yr of age. The uninfected PHS from TMMC had been taken into captivity prior to weaning and were at least 6 wk of age when the serum was taken.

Extracts from three additional nematode genera were also run on the gels to determine species specificity of the Ig binding pattern in NES and PHS. *Parafilaroides* (from NES, PHS, and California sea lion [CSL, *Zalophus californianus*]); *Anisakis* (from NES); and *Pseudoterranova* (from NES) were used because these genera also infect NES and PHS in California. These were probed with serum from two of the NES infected with *O. circumlitus* that were 2–6 mo of age, one of the uninfected NES, and one of the infected PHS of 2–6 mo. In addition, two 2- to 6-mo-old NES infected with *O. circumlitus* and one of the 4- to 6-mo-old infected PHS were tested with all of the extracts except *Parafilaroides* sp. from PHS, which was available only in very small quantities.

RESULTS

Immunoblots of *O. circumlitus* extracts

There was individual variation in intensity and location of bands on immunoblots among seals. Despite this variation, seals of different species and age classes had distinct patterns. The principle differences between patterns in these seal classes are summarized in Table 1. Intensity of band staining for each species and age class was graded visually on a 5-point scale from 0 for no staining to 4 for very intense staining. This analysis was performed for each individual seal and the mean banding intensity for each species and age class indicated in the table.

Immunoblots using sera from infected NES that were 6 mo of age or younger had a strong band of about 28 kDa that was detected most intensely in the female reproductive tract of the nematode (Fig. 1a; Table 1). A band of this approximate size was also present to a lesser degree in all other tissues except body wall fraction F3. The 9-mo-old NES had a weakly stained band of this size. When a gradient gel was probed with serum from a 4- to 6-mo-old animal, this band was shown to be composed of a strongly staining band of 28 kDa and a band of 26 kDa that stained less strongly in all the aforementioned tissues, except the ES glands. The band in the ES glands was composed of a triplet of bands of 27, 26, and 24 kDa. All infected animals ≤9 mo of age that had antibodies binding strongly to the female reproductive tract also had antibodies binding to all of the nematode tissues. However, no one tissue appeared to be more intensely stained than the others. None of the uninfected NES sera tested had Ig binding to the 28 kDa band (Table 1) or the 26 kDa band. Sera from two uninfected seals did not contain detectable antibodies recognizing any of the nematode tissues. The serum from the third uninfected seal produced faint smears on the gel without distinct

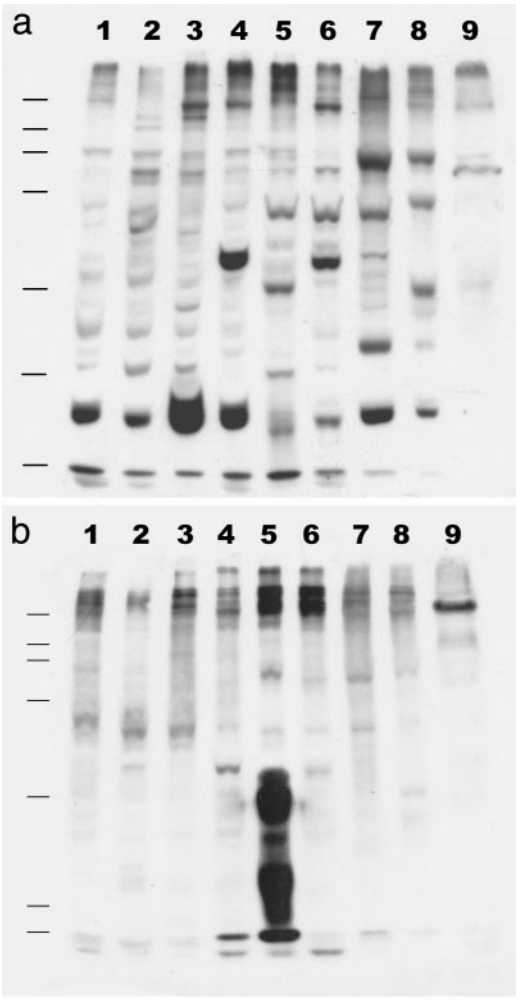

FIGURE 1. (a) Blot of *Otostrongylus circumlitus* tissues probed with serum from a 6-mo-old *O. circumlitus*–infected northern elephant seal (NES) showing characteristic banding pattern for this age class. Note the strongly stained 28 kDa band in the female reproductive tract (lane 3). Lines at left of figure show position of 200, 116.25, 97.4, 66.2, 45, 31, and 21.5 kDa markers. Lanes contain extracts of the following *O. circumlitus* tissues. Lane 1: whole male. Lane 2: male reproductive tract. Lane 3: female reproductive tract. Lane 4: whole female. Lane 5: excretory-secretory (ES) glands. Lane 6: intestine. Lanes 7–9: body wall extracts. Lane 7: F1. Lane 8: F2. Lane 9: F3. (b) Blot of *O. circumlitus* tissues probed with serum from a 1-yr-old *O. circumlitus*–infected NES showing characteristic banding pattern for this age class. Note the strongly stained ES glands (Lane 5). Lines at left of figure show position of 200, 116.25, 97.4, 66.2, 45, 31, and 21.5 kDa markers. Lanes contain extracts of the following *O. circumlitus* tissues. Lane 1: whole male. Lane 2: male reproductive tract. Lane 3: female reproductive tract. Lane 4: whole female. Lane 5: ES glands. Lane 6: intestine. Lanes 7–9: body wall extracts. Lane 7: F1. Lane 8: F2. Lane 9: F3.

bands, and that of the fourth uninfected seal had one strongly stained band of about 97 kDa in the lanes containing whole adult *O. circumlitus* and a few weakly stained bands in the body wall fractions. In 1 yr and older NES, intensity of the 28 kDa band varied from nonexistent to strong. All older animals contained antibodies to all *O. circumlitus* tissues; however, the ES glands appeared to be most strongly stained (Fig. 1b; Table 1). This tissue did not appear to be as strongly stained in the younger NES (Fig. 1a; Table 1).

Two of the 12 infected PHS tested had Ig binding patterns that were too faint for analysis. For all PHS in which antibodies were detected, Ig bound to all of the nematode tissues tested. However, in common with the ≥1-yr-old infected NES, eight of the remaining 10 infected PHS had strong binding to the ES glands (Table 1). Seven of these animals lacked the antibodies to the 28 kDa band in the female reproductive tract that were seen in young NES, but a very faint band in this position was detected in one animal. The antibody binding pattern of the two remaining PHS was similar to that of the young NES (a reasonably strong 28 kDa band in the female reproductive tract and binding to the ES glands at the same approximate strength as the other tissues). The banding pattern of the >4-yr-old animal was similar to that of the majority of the younger PHS. This PHS appeared to contain high levels of Ig that recognized the ES glands, but binding to the 28 kDa band of the female reproductive tract was not detected. Sera of the uninfected animals did not appear to contain high levels of Ig recognizing the ES glands, with the exception of one animal that had a strong band of about 28 kDa. In this animal Ig appeared to bind strongly to a band of this size in all tissues, except body wall fraction F3. Furthermore, the band was of similar intensity in the female reproductive tract, whole *O. circumlitus,* male reproductive tract, ES glands, and gut. Specifically, in the four

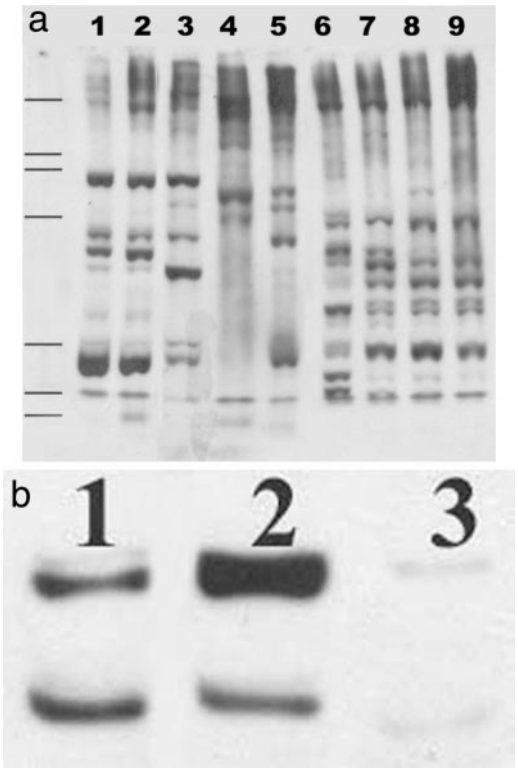

FIGURE 2. (a) Blot of nematode tissues probed with serum from an *Otostrongylus circumlitus*–infected 6-mo-old Pacific harbor seal (PHS). Lines at left of gel show position of 200, 116.25, 97.4, 66.2, 45, 31, and 21.5 kDa markers. Lanes contain extracts of the following nematode tissues. Lane 1: whole male *O. circumlitus*. Lane 2: whole female *O. circumlitus*. Lanes 3–5: *Parafilaroides* sp. Lane 3: From northern elephant seal (NES). Lane 4: from Pacific harbor seal. Lane 5: from California sea lion. Lane 6: *Pseudoterranova* sp. larva. Lanes 7–9: *Anisakis* sp. Lane 7: larva. Lane 8: adult male. Lane 9: adult female. (b) Gradient gel blotted and probed with serum from an *O. circumlitus*–infected 6-mo-old NES. Only the 28 and 26 kDa bands are shown. Lane 1: extract of whole female *O. circumlitus*. Lane 2: extract of *O. circumlitus* female reproductive tract. Lane 3: extract of whole *Parafilaroides* sp. from NES.

uninfected controls from Sea World, binding was not detected for one animal, one had nonspecific faint binding, and one had very faint binding (equivalent to an animal infected with *O. circumlitus* that had Ig levels that were too low for analysis). The binding was so weak in these animals that they were scored as zero. The last animal, however, had the 28 kDa band previously mentioned and a band of about 60 kDa that was detected in all tissues except two of the body wall fractions. Several bands were detected in all tissues for one of the PHS from TMMC, but no 28 kDa band was seen. In the other uninfected PHS from TMMC, some high molecular weight bands were detected in the whole *O. circumlitus* and body wall fractions, and there were a few bands in the ES glands.

Immunoblots of different nematode genera

Banding patterns in immunoblots using phocid sera against whole nematode extracts varied between species of nematode (Fig. 2a). *Otostrongylus* was easily distinguished from the other genera tested. Immunoblot patterns were quite different among the *Parafilaroides* sp. from different host species. Immunoblot patterns within the anisakid group were, however, quite similar, with *Pseudoterranova* sp. being the most distinct. When blots were probed with sera from juvenile PHS, the 28 kDa band was not seen for any of the nematode genera (Fig. 2a). In immunoblots using juvenile NES sera, however, *Parafilaroides* lanes showed antibodies to a protein of about the same molecular weight as the intensely staining 28 kDa protein in *O. circumlitus*. This band appeared to be strongest in the *Parafilaroides* sp. from NES. To test whether this is the same protein as the 28 kDa band of *O. circumlitus* detected with serum from juvenile NES infected with *O. circumlitus*, *Parafilaroides* sp. from NES was run on a gradient gel and compared with extracts of *O. circumlitus* whole nematodes and female reproductive tract by immunoblotting with serum from juvenile NES infected with *O. circumlitus* (Fig. 2b). Despite banding intensity being much weaker for *Parafilaroides* sp. than for *O. circumlitus*, the band was shown to be the same size in the two genera. No antibodies to extracts from the different genera were detected in the uninfected NES.

DISCUSSION

We successfully used Western blot methodologies to examine and compare differences in nematode tissue-specific serum antibodies between NES and PHS of different ages. Although Western blots are not quantitative, results of this study suggest that NES ≤9 mo of age that were infected with *O. circumlitus* had Ig binding at a similar strength for all *O. circumlitus* tissues. However, the host would be expected to respond most strongly to the tissues and secretions to which it is exposed. Immunoglobulin from infected animals was thus expected to bind strongly to the cuticle because it is the external surface of the nematode and is sloughed during molting. Also, adult *O. circumlitus* have large and highly active ES glands (Elson-Riggins et al., 2002); thus we expected these to bind Ig strongly in infected animals. However, only PHS and older NES had strong binding to these antigens. Binding of Ig to internal antigens was unexpected.

Host response to internal antigens may indicate parasite death or molt. Kennedy et al. (1989) showed that immune responses to secreted and somatic antigens of parasitic nematodes occur. A 14 kDa protein that occurred in ES secretions and pseudocoelomic fluid (perienteric fluid as used by Kennedy et al., 1989) of *Ascaris* (Ascarida) had a homologue that occurred in the pseudocoelomic fluid only of *Toxocara canis* (Ascarida; Kennedy et al., 1989). This protein was immunoprecipitated from both *Ascaris* and *T. canis* using antiserum to *Ascaris* ES products and larvae, but not using antiserum to *T. canis* ES secretions or larvae. Further evidence that the host may not always be exposed to internal antigens comes from work on *Anisakis simplex*. Hosts infected with *A. simplex* larvae did not respond to the 14 kDa protein, but those exposed to *A. simplex* homogenate did (Kennedy et al., 1988). *Anisakis simplex* was shown to contain a homologue of the 14 kDa *Ascaris* protein, but the host did not have an IgG response. In addition, hosts infected with *A. simplex* had a stronger immune response to ES components than somatic materials.

In light of the current results and the fact that clinical signs and lesions associated with *O. circumlitus* infection can be severe in juvenile NES, it is likely that many of these parasites die and disintegrate in this host. From 1992 to 1995, 37% of NES weanling deaths and 12% of PHS deaths at TMMC were associated with *O. circumlitus* (Gulland et al., 1997). Juvenile NES often died before the female nematodes started shedding larvae in the feces. High numbers of dead and disintegrating parasites could result from the severe inflammatory response to migrating larvae observed in juvenile NES (Gulland et al., 1997). In contrast, yearling NES often shed *O. circumlitus* larvae in feces and rarely show clinical signs associated with the infection (Gulland, pers. obs.).

Although conclusions regarding the quantitative aspects of the humoral responses were limited by our techniques, this work suggested that NES ≥1 yr old infected with *O. circumlitus* and the majority of PHS infected with *O. circumlitus* had a stronger immune response to the ES glands of *O. circumlitus* than the other tissues. Thus, the different age classes of NES seem to respond differently to the ES glands. It is not possible, however, to determine from the current results whether the older NES are responding to additional proteins in the ES glands or whether they are producing a stronger immune response to the same proteins as the younger NES. Assays such as enzyme-linked immunosorbent assays (ELISA) are needed to quantify the response. A further limitation of this study is that the Ig detected was mostly IgG, rather than IgE. This is a consequence of the use of protein A to detect Ig binding. Protein A was used because it is a readily available reagent and has been previously used for phocid Ig detection. However, there are likely to be species differences in the amount and type

of Ig detected by this method. Ideally, IgE should be investigated in future studies as this class of Ig is commonly involved in host reaction to parasites (Kennedy, 2000).

Four hypotheses may account for the difference in Ig binding patterns between the NES age classes. First, older NES may be better able to respond to the proteins secreted by the ES gland than younger NES, this response being protective. Onderka (1989) suggested that the peribronchial inflammatory reaction of ringed seals to *O. circumlitus* might be a response to secretions from the excretory pore. This author reported that the anterior 5–10 mm of the nematodes were embedded in the peribronchial tissue. Thus, since the excretory pore is usually 437–617 μm from the anterior of the nematode (Elson-Riggins et al., 2001), it is possible that secretions from the ES glands are responsible for the host response. A second explanation is that younger NES may have such a strong response to the parasites that the nematodes do not survive to the stage where the glands are actively secreting; that is, the majority of the parasites die and disintegrate in the host and the host usually dies from the infection. Thus, the older NES may have an increased Ig response to the ES glands because they are exposed to higher concentrations of these proteins as the worms mature. The younger NES would still be exposed to the contents of the ES glands when the parasites die and disintegrate, but these proteins would be present in lower concentrations, hence the lesser immune response. A third possibility is that there is a sequential recognition of ES antigens over time. This might occur if the parasite undergoes antigenic switching. Juvenile NES tend to be infected with smaller and more immature worms than PHS and NES 1 yr and older (Gulland et al., 1997). Thus, small nematodes may not be secreting the same ES antigens as larger nematodes. The fourth potential explanation is that there is individual variation in response to *O. circumlitus* antigens, irrespective of age. If individuals responding to a suite of antigens excluding the strong response to the ES glands died as juveniles, whereas individuals responding strongly to the ES glands survived infection, a change in response with age would be observed. Determination of which explanation is most likely will require longitudinal studies of the immune response of individual seals to *O. circumlitus* over time.

The majority of the infected PHS had an Ig binding pattern to *O. circumlitus* that appeared to be similar to that of the 1- to 2-yr-old NES. Immunoglobulin bound to all nematode tissues, but binding was strongest for the ES glands. Pacific harbor seals appear to be better adapted to *O. circumlitus* infection than juvenile NES (Gulland et al., 1997). Gulland et al. (1997) reported that PHS shed larvae in the feces much earlier than NES, beginning in June as compared with August for NES. Deaths took place much earlier in NES, occurring from April to August during the prepatent period, compared with June to December during the patent period for PHS. However, once NES reach approximately 1 yr of age, they are more likely to survive the infection.

A significant immune response to the parasite was not detected in two of the PHS that were infected with *O. circumlitus.* It is not known whether this caused them to succumb to the infection any faster than the other PHS. Interestingly, one of these animals had 10 *O. circumlitus* in the right ventricle and pulmonary artery and the other had 50 *O. circumlitus* in the pulmonary artery. A fecal examination on one of these animals was negative for *O. circumlitus* larvae. Mortality of PHS from *O. circumlitus* during the prepatent period is not typical (Gulland et al., 1997). Two other infected PHS had a similar Ig binding pattern to that of the juvenile NES. One of these two PHS succumbed to DIC as a result of the infection, which is a more typical host-parasite reaction in juvenile NES than in PHS (Gulland et al., 1997). It is therefore possible that this Ig binding

pattern may be typical of disease during the prepatent period. This animal had 30 adult *O. circumlitus* in the vena cava. On necropsy, the second PHS had 24 adult *O. circumlitus* in the pulmonary artery. Arteritis of the colon was also evident. This may have resulted from aberrant parasite migration. Further work on individual cases is required to establish whether there is any link between the Ig binding pattern, location of the nematodes, prepatent period, and severity of the infection.

The response of uninfected PHS was much less uniform than the uninfected NES. However, strong Ig binding to the ES glands was not detected in any of these animals. No difference in the banding pattern was detected between the older infected PHS tested and the younger infected PHS tested. If the ES glands are an important source of antigens that are deleterious to the host, then there is probably no need for a shift in pattern with age since young PHS have a banding pattern similar to older NES. It would be of benefit, however, to test a greater number of older PHS.

It was possible to distinguish *O. circumlitus* from the other nematode genera tested by running extracts of whole nematodes on gels and probing with seal serum. Furthermore, all juvenile NES tested that were infected with *O. circumlitus*, unlike uninfected NES, had Ig that bound to a 28 kDa protein that was dominant in the female nematode reproductive tract. Immunoglobulins binding to this protein were also detected in the serum of some of the infected 1- to 2-yr-old NES and in serum of three infected PHS. In addition, one uninfected PHS also had Ig that bound to a protein of the same size. The intensity of the band using serum from this animal did not, however, appear to be greater for the female reproductive tract of *O. circumlitus* than the other nematode tissues. Because mortality of NES infected with *O. circumlitus* occurs during the prepatent period, ante mortem diagnosis of infections is not currently possible in this host species. Due to the intensity of the 28 kDa band in the female reproductive tract of *O. circumlitus* in Western blots probed with serum from juvenile infected NES and its absence in uninfected NES, we suggest that this protein may be a suitable marker for an antibody-based diagnostic test for *O. circumlitus* in these animals. There was, however, a band of this size present in extracts of *Parafilaroides* sp. in blots probed with serum from infected juvenile NES. Further work is therefore required to determine whether this is the same protein in both genera and hence its suitability as a marker for *O. circumlitus* infection. However, in contrast to *O. circumlitus* infection, juvenile NES infected with *Parafilaroides* sp. shed larvae in their feces when clinical signs occur. Therefore, even if this protein proves to be the same in both nematode genera, it may be possible to use the 28 kDa band as a marker of *O. circumlitus* infection in conjunction with a fecal exam for *Parafilaroides* larvae in clinical infections.

In conclusion, there are marked differences in Ig binding patterns to antigens of *O. circumlitus* among NES of different age classes and between seal species. Further studies are required to quantify antibody binding and to determine association between differences in immune response and clinical signs and lesions.

ACKNOWLEDGMENTS

Samples were collected at The Marine Mammal Center and Sea World, and we are indebted to the staff and volunteers of these institutions for their assistance. We also thank M. Dailey for nematode collection and T. Hoodbhoy, J. E. Feugate, L. Wong, M. Martins-Green, and D. King for technical advice. We especially thank B. Aldridge and P. Talbot for technical advice, helpful discussions, and for reviewing the manuscript. We also thank I. Kaloshian and J. Baldwin for reviewing the manuscript. J. Elson-Riggins was partially supported by a GAANN fellowship from the US Department of Education. This research was also funded by the following sources at the University of California, Riverside: the Newell Fund of the Department of Biology, research assistantships from the Department of Nematology,

a Dissertation Research Grant from the Graduate Division, and a Janet M. Boyce Award from the College of Natural and Agricultural Sciences.

LITERATURE CITED

BERGERON, E., L. M. MEASURES, AND J. HUOT. 1997. Experimental transmission of *Otostrongylus circumlitus* (Raillet, 1899) (Metastrongyloidea: Crenosomatidae), a lungworm of seals in eastern arctic Canada. Canadian Journal of Zoology 75: 1364–1371.

DOUCET, J. P., AND J. M. TRIFARÓ. 1988. A discontinuous and highly porous sodium dodecyl sulphate–polyacrylamide slab gel system of high resolution. Analytical Biochemistry 168: 265–271.

ELSON-RIGGINS, J. G. 2002. Characterization of *Otostrongylus circumlitus,* a lungworm from seals, and host-parasite interaction in harbor and northern elephant seals. PhD Dissertation, University of California, Riverside, California, USA, 156 pp.

———, L. AL-BANNA, E. G. PLATZER, AND I. KALOSHIAN. 2001. Characterization of *Otostrongylus circumlitus* from Pacific harbor seals and northern elephant seals. Journal of Parasitology 87: 73–78.

GULLAND, F. M. D., L. WERNER, S. O. NEILL, L. J. LOWENSTINE, J. TRUPKIETWITZ, D. SMITH, B. ROYAL, AND F. STRUBEL. 1996. Baseline coagulation assay values for northern elephant seals (*Mirounga angustirostris*) and disseminated intravascular coagulation in this species. Journal of Wildlife Diseases 32: 536–540.

———, K. BECKMEN, K. BUREK, L. LOWENSTINE, L. WERNER, T. SPRAKER, M. DAILEY, AND E. HARRIS. 1997. Nematode (*Otostrongylus circumlitus*) infestation of northern elephant seals stranded along the central California coast. Marine Mammal Science 13: 446–459.

KENNEDY, M. W. 2000. Immune response to *Anisakis simplex* and other ascarid nematodes. Allergy 55(Suppl. 59): 7–13.

———, J. TIERNEY, P. YE, F. A. MCMONAGLE, A. MCINTOSH, D. MCLAUGHLIN, AND J. W. SMITH. 1988. The secreted and somatic antigens of the third stage larva of *Anisakis simplex,* and antigenic relationship with *Ascaris suum, Ascaris lumbricoides,* and *Toxocara canis.* Molecular and Biochemical Parasitology 31: 35–46.

———, F. QURESHI, E. M. FRASER, M. R. HASWELL-ELKINS, D. B. ELKINS, AND H. V. SMITH. 1989. Antigenic relationships between the surface-exposed, secreted and somatic materials of the nematode parasites *Ascaris lumbricoides, Ascaris suum,* and *Toxocara canis.* Clinical and Experimental Immunology 75: 493–500.

LAEMMLI, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685.

MAIZELS, R. M., M. L. BLAXTER, B. D. ROBERTSON, AND M. E. SELKIRK. 1991. Parasite antigens, parasite genes. A laboratory manual for molecular parasitology. Cambridge University Press, Cambridge, England, 224 pp.

MEASURES, L. N. 2001. Lungworms of marine mammals. *In* Parasitic diseases of wild mammals, W. M. Samuel, M. J. Pybus, and A. A. Kocan (eds.). Iowa State University Press, Ames, Iowa, pp. 279–300.

ONDERKA, D. K. 1989. Prevalence and pathology of nematode infections in the lungs of ringed seals (*Phoca hispida*) of the western arctic of Canada. Journal of Wildlife Diseases 25: 218–224.

RIEDMAN, M. 1990. The pinnipeds: Seals, sea lions and walruses. University of California Press, Berkeley, California, 439 pp.

VERCRUYSSE, J., A. SALOMEZ, A. ULLOA, M. ALVINERIE, A. OSTERHAUS, AND T. KUIKEN. 2003. Efficacy of ivermectin and moxidectin against *Otostrongylus circumlitus* and *Parafilaroides gymnurus* in harbor seals (*Phoca vitulina*). The Veterinary Record 152: 130–134.

Received for publication 9 February 2004.